# *ACETYLCHOLINESTERASE, II. CRYSTALLIZATION, ABSORPTION SPECTRA, ISOIONIC POINT**

By Walo Leuzinger, A. L. Baker,† and Elsa Cauvin

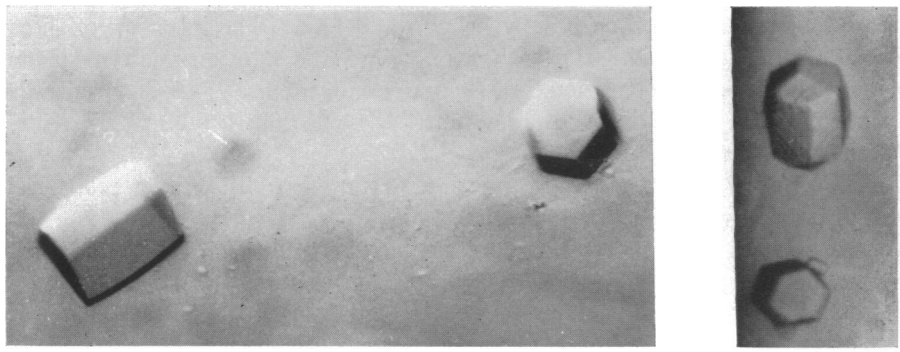
DEPARTMENTS OF BIOCHEMISTRY AND NEUROLOGY,
COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

*Communicated by David Nachmansohn, December 22, 1967*

The paramount role of acetylcholinesterase (ACh-esterase) in the control of electrical activity of excitable membranes of nerve and muscle cells appears well established.[1-3] In view of its vital function in nerve impulse conduction, the enzyme has been intensively studied since it was first extracted, in 1938, from electric tissue.[4] In the early 1940's a several-hundredfold purification of this enzyme was achieved;[5] however, the amounts of pure protein available, while adequate for kinetic studies, for the analysis of the molecular groups in the active site and other related studies, did not permit an investigation of the protein structure and its properties. The advances of macromolecular chemistry in the last decade, especially the possibility of exploring tridimensional structures, made apparent the desirability of obtaining the enzyme in sufficiently large amounts for carrying out such studies. A large-scale purification was started about four years ago, and a successful and readily reproducible procedure was described in a preceding paper:[6] 60–70 mg pure protein were obtained from 10 kg of electric tissue. Evidence for the purity of the enzyme and an amino acid analysis were also reported. In a footnote added in proof, a crystallization of the enzyme was announced. In the present paper the growth of the crystals, the absorption spectra, extinction coefficient, and the isoionic and isoelectric points are described.

*Materials and Methods.*—ACh-esterase, with a specific activity of approximately 750 mmoles ACh hydrolyzed per milligram of protein per hour, has been used throughout the experiments described here. The reaction mixture in which the specific activity was tested contained 0.02 *M* $MgCl_2$, 0.01% recrystallized bovine albumin, and $2.7 \times 10^{-3}$ *M* acetylcholine. The presence of $Mg^{++}$ has been found essential for full enzyme activity; in the absence of this ion, the activity decreased by 40%. The effect of $Mg^{++}$ has been previously discussed.[7, 8] A more detailed evaluation of the effects of mono- and divalent cations on the activity will be described elsewhere. A radiometer automatic titrator was used for determining the enzyme activity. The protein concentrations were measured by the optical absorption at 280 mμ and by micro-Kjeldal methods. The UV-absorption spectra were recorded with a Cary 14 spectrophotometer.

*Results.—Crystallization:* When a concentrated solution of pure enzyme protein (0.5–1%) is kept in 35 per cent ammonium sulfate solution at 4°C, crystals are formed within about two to four days.[6] These crystals have a length of about 10 μ. Growth of the crystals has been obtained in the following way: saturated ammonium sulfate is slowly added to a solution of 0.5–1 per cent pure enzyme in 0.06 *M* potassium phosphate buffer, pH 6.85, until a slight turbidity occurs. This represents approximately 35 per cent saturation. The solution is then clarified by centrifugation and slowly concentrated over a period of two to three months by evaporating water through a thin capillary tube. Using this method, crystals of 150 μ length and 120 μ width have been isolated. The



A B

FIG. 1.—Crystals of ACh-esterase. Magnification 100 ×. (*A*) Regular hexagonal prisms; (*B*) possibly pyramidal termination.

crystals are extremely well formed. The most common form of growth observed is short, thick prisms of regular hexagonal cross section (Fig. 1), a form compatible with true hexagonal symmetry. The complex pyramidal termination shown by some crystals, however, may imply a crystal system of lower order. The crystals exhibit low birefringence so that it is not possible at present to determine whether they are uniaxial or biaxial.

*Absorption spectra:* Figure 2 illustrates the absorption spectra of ACh-

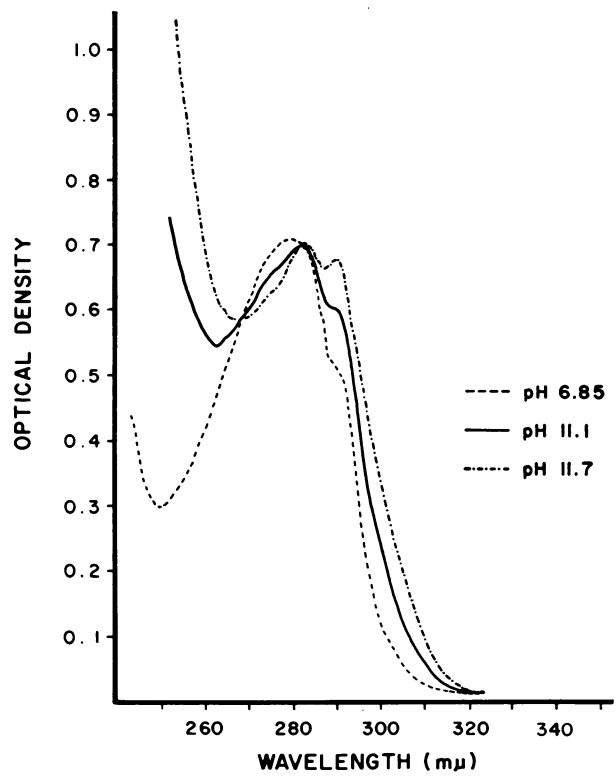



FIG. 2.—UV-absorption spectra of ACh-esterase in 0.02 *M* potassium phosphate at pH 6.85, 11.1, 11.7.

esterase at various pH values between 6.85 and 11.7. The intensity of the absorption at 280 mμ stays almost the same when the solution is altered from a neutral to an alkaline condition, but the maximum intensity, as shown in Figure 2, undergoes a slight bathochromic shift. Two isosbestic points are seen at 268 mμ and 282 mμ.

*Extinction coefficient:* A determination was made of the direct relationship between the dry weight of ACh-esterase and optical density at 280 mμ. The enzyme appears to have a very low solubility in distilled water and therefore there is insufficient protein in the solution for an optical density determination at 280 mμ. For this reason, enzyme was dissolved in either 0.02 *M* $(NH_4)_2CO_3$ or 0.02 *M* $CH_3COONH_4$ solution and dialyzed over a period of two days against the same salt solutions. The dialyzed enzyme was centrifuged for 5 minutes at 30,000 rpm, and subsequently the optical density was measured at 280 mμ. An aliquot was lyophilized, heated at 130°C for 20 minutes, and finally cooled in a desiccator over $P_2O_5$. This procedure was repeated daily until the weight was constant. A determination of the dry weight was thus obtained, and the extinction coefficient was found to be $E^{1\%}_{280\ m\mu} = 16.1$.

*Solubility:* The solubility of ACh-esterase in 0.01 *M* NaCl solution has been determined in the region of minimum solubility. This region has been approached from both sides by adjusting 0.1 per cent enzyme solutions to the desired pH with NaOH or HCl. After standing for ten minutes the solutions were centrifuged and the protein concentration of the supernatant measured. The resulting data in the region of minimum solubility are shown in Figure 3. Minimum solubility occurs at pH 5.1, which would indicate that the isoelectric point lies close to, or at, that point.

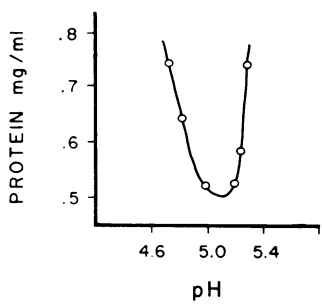



FIG. 3.—Solubility of ACh-esterase in 0.01 *M* NaCl solution as function of pH.

*Isoionic point:* The isoionic point of ACh-esterase was found by dialyzing a protein solution against distilled water. Complete removal of salts was obtained by passing the solution through a column of mixed bed resin (Bio-Rad AG 501-X8); the pH value of the eluate was 5.35.

*Discussion.*—Crystals of ACh-esterase have so far been grown to a length of 150 μ and to a width of 120 μ. It is hoped that a size will soon be obtained which will permit the initiation of studies by X-ray crystallography. In combination with investigations of the subunits, amino acid sequences, and other aspects, a better insight in the properties of ACh-esterase may then be obtained.

In the last few years, electron microscopy studies combined with histochemical staining techniques have borne out the idea proposed more than 20 years ago on the basis of indirect biochemical studies that the enzyme is structurally bound to membranes.[9–14] Membranes to which ACh-esterase is attached have been isolated from electric tissue of *Electrophorus electricus*.[15] Some characteristics of this membrane-bound enzyme as compared to those of the enzyme

in solution have been recently reported.[16] Moreover, the monocellular electroplax preparation developed by Schoffeniels[17] and greatly refined during the last decade[18] permits study of the enzyme (and related proteins) on a cellular level.[19-24] Contemporary biochemistry has made it increasingly apparent that characterization of an enzyme requires studies not only in solution but also within the frame of the natural macromolecular assembly of the cell, in view of the many cooperative effects, regulatory and control mechanisms, on which the activity of an enzyme depends. The availability of crystals and of pure protein in adequate amounts in combination with the other preparations has thus opened the possibility of studying the characteristics of acetylcholinesterase on a cellular, subcellular, and molecular level.

The authors wish to thank Professor David Nachmansohn for his advice and interest. Miss Evelyn Kissin provided able assistance.

* This work was supported in part by the U.S. Public Health Service grant NB-03304 and by the National Science Foundation grant GB 4844.

† Technical Director, Worthington Biochemical Corporation, Freehold, New Jersey.

[1] Nachmansohn, D., *Chemical and Molecular Basis of Nerve Activity* (New York: Academic Press, 1959), p. 235.

[2] Nachmansohn, D., in *New Perspectives in Biology*, ed. M. Sela, BBA Library (Amsterdam: Elsevier, 1964), vol. 4, p. 176.

[3] Nachmansohn, D., *Ann. N.Y. Acad. Sci.*, **137**, 877 (1966).

[4] Nachmansohn, D., and E. Lederer, *Bull. Soc. Chim. Biol.*, **21**, 797 (1939).

[5] Rothenberg, M. A., and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

[6] Leuzinger, W., and A. L. Baker, these PROCEEDINGS, **57**, 446 (1967).

[7] Nachmansohn, D., *Nature*, **145**, 513 (1940).

[8] Friess, S. L., I. B. Wilson, and E. Cabib, *J. Am. Chem. Soc.*, **76**, 5156 (1954).

[9] Torack, R. M., and R. J. Barrnett, *Exptl. Neurol.*, **6**, 224 (1962).

[10] Bloom, F. E., and R. J. Barrnett, *J. Cell Biol.*, **29**, 475 (1966).

[11] Brzin, M., V. Tennyson, and P. E. Duffy, *J. Cell Biol.*, **31**, 215 (1966).

[12] Schlaepfer, W. W., and R. M. Torack, *J. Histochem. Cytochem.*, **14**, 369 (1966).

[13] Novikoff, H. B., N. Quintana, H. Villaverde, and R. Forschirm, *J. Cell Biol.*, **29**, 525 (1966).

[14] Brzin, M., these PROCEEDINGS, **56**, 1560 (1966).

[15] Karlin, A., *J. Cell Biol.*, **25**, 159 (1965).

[16] Silman, H. I., and A. Karlin, these PROCEEDINGS, **58**, 1664 (1967).

[17] Schoffeniels, E., *Biochim. Biophys. Acta*, **26**, 585 (1957).

[18] Higman, H. B., T. R. Podleski, and E. Bartels, *Biochim. Biophys. Acta*, **75**, 187 (1963).

[19] Bartels, E., and D. Nachmansohn, *Biochem. Z.*, **342**, 359 (1965).

[20] Podleski, T. R., these PROCEEDINGS, **58**, 268 (1967).

[21] Karlin, A., and E. Bartels, *Biochim. Biophys. Acta*, **126**, 525 (1966).

[22] Karlin, A., *J. Theoret. Biol.*, **16**, 306 (1967).

[23] Podleski, T., and J.-P. Changeux, *Science*, **157**, 1579 (1967).

[24] Changeux, J.-P., T. R. Podleski, and L. Wofsy, these PROCEEDINGS, **58**, 2063 (1967).